Fully Human Monoclonal Antibodies from Antibody Secreting Cells after Vaccination with Pneumovax®23 are Serotype Specific and Facilitate Opsonophagocytosis

Kenneth Smith[*,a], **Jennifer J. Muther**[a], **Angie L. Duke**[a], **Emily McKee**[a], **Nai-Ying Zheng**[b], **Patrick C. Wilson**[b], and **Judith A. James**[a,c]

[a]Oklahoma Medical Research Foundation, Arthritis and Clinical Immunology Research Program, 825 NE 13th Street, Oklahoma City, OK 73104

[b]University of Chicago, Department of Medicine, 924 E 57th St., Chicago, IL 60637

[c]University of Oklahoma Health Sciences Center, Departments of Medicine, Pathology and Microbiology and Immunology, 1100 N. Lindsay, Oklahoma City, OK 73104

Abstract

B lymphocyte memory generates antibody-secreting cells (ASCs) that represent a source of protective antibodies that may be exploited for therapeutics. Here we vaccinated four donors with Pneumovax23 and produced human monoclonal antibodies (hmAbs) from ASCs. We have cloned 137 hmAbs and the specificities of these antibodies encompass 19 of the 23 serotypes in the vaccine, as well as cell wall polysaccharide (CWPS). Although the majority of the antibodies are serotype specific, 12% cross-react with two serotypes. The Pneumovax23 ASC antibody sequences are highly mutated and clonal, indicating an anamnestic response, even though this was a primary vaccination. Hmabs from 64% of the clonal families facilitate opsonophagocytosis. Although 9% of the total antibodies bind to CWPS impurity in the vaccine, none of these clonal families showed opsonophagocytic activity. Overall, these studies have allowed us to address unanswered questions in the field of human immune responses to polysaccharide vaccines, including the cross-reactivity of individual antibodies between serotypes and the percentage of antibodies that are protective after vaccination with Pneumovax23.

Keywords

Antibody secreting cells; B cell memory; human monoclonal antibodies; Pneumovax; *Streptococcus pneumoniae*

Introduction

Streptococcus pneumoniae is a ubiquitous human pathogen that causes a range of clinical infections, such as otitis media, pneumonia, meningitis, and bacteremia. The more serious manifestations are especially virulent in immunocompromised and elderly individuals. More than 90 different *S. pneumoniae* serotypes have been characterized, each having a different

[*]**Corresponding Author:** Kenneth Smith, Ph.D., 825 NE 13th Street, Oklahoma City, OK 73104, (405)271-3275, smithk@omrf.org.

capsular polysaccharide structure. These polysaccharides are immunogenic in adults, and the Pneumovax23 vaccine consists of a cocktail of 23 of the most common and/or virulent *S. pneumoniae* strains. The vaccine is recommended for everyone over the age of fifty, as well as all immunocompromised individuals, to improve seroprotection against these strains. The serology of the response to Pneumovax23, as well as the conjugate vaccine Prevnar (used to immunize children), has been studied in depth with regard to the humoral polyclonal IgG and IgA responses in both sera and saliva (Antilla et al., 1999; Nieminen et al., 1998; Nieminen et al., 1998). The memory and antibody secreting cell (ASC) response to these vaccines has also been previously explored on a cellular level with B cell ELISpot assays and flow cytometry (Nieminen et al., 1998; Clutterbuck et al., 2006; Baxendale et al., 2010), and the presence of both responses after vaccination is now well established. However, utilizing ASCs to produce human monoclonal antibodies provides a novel means to fully elucidate the recall response to pathogen serotypes after vaccination, and provides a window to explore the evolution of past responses.

Antibodies that cross-react with two or more pneumococcal polysaccharides are present in sera both pre- and post-immunization (Lee, C.-J. et al., 1984; Soininen et al., 2000); however, whether this is due to single antibody specificities that are capable of cross-reacting or due to broad polyclonal antibody specificities is not known. Thus, we reasoned that examining this response at the monoclonal level would provide new insight into many aspects of the anti-polysaccharide immune response.

To explore these questions on a per antibody basis we vaccinated patients with the Pneumovax23 vaccine, generated and characterized large numbers of high affinity human monoclonal antibodies to the *S. pneumoniae* serotypes and cell wall polysaccharide (CWPS) present in the vaccine. Although human monoclonal antibodies to *S. pneumoniae* have been produced in the past (Baxendale and Goldblatt, 2006; Baxendale et al., 2000; Zhou et al., 2002; Zhou et al., 2004), these previous studies have been limited by two factors: one, they employed Fab expression library screens and two, they employed random production of hybridomas. In addition, previous studies have either focused on one serotype (6B and 23F) or have utilized vaccination with the conjugate vaccine Prevnar that consists of only seven capsular serotypes. In contrast, our technique provides a more cross-sectional characterization of the anti-polysaccharide response at one particular point in time, seven days post vaccination. Prior to monoclonal antibody isolation, ASCs were sorted; thus, every cell used to clone an antibody arose from a memory response to this particular vaccination. This system allows us to shed light on a number of as yet still unanswered questions in the field of polysaccharide immune responses. In this report, we have specifically addressed the percentage of human monoclonal polysaccharide antibodies that cross-react between different serotypes, bind to CWPS, and most importantly facilitate opsonophagocytosis.

Materials and Methods

Immunization and donors

Four donors received Pneumovax23 (Merck, Whitehouse Station, NJ) as standard of care vaccination based upon their age or diagnosis of systemic lupus erythematosus (SLE). Donors PVAX1 and PVAX2 were both Caucasian and without known autoimmune disease; age 62, male, and 61, female, respectively. Two donors were SLE patients: PVAX3, an African American male, age 47, and PVAX4, a Caucasian female, age 45. All protocols were approved by the OMRF Internal Review Board, and patients consented to participate in this study. Blood was drawn (~40–60 ml) into ACD tubes (BD, Franklin Lakes, NJ) by venipuncture seven days post vaccination and was stored no longer than 18 hours before processing.

Cell isolation and flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated from fresh blood using lymphocyte separation medium (Cellgro, Manassas, VA) and suspended in 2% inactivated fetal calf serum in PBS. Cells were then counted and stained within two hours of the isolation. Antibodies used for the staining were anti-CD3 and anti-CD20 conjugated to FITC, anti-CD38 conjugated to APC-Cy5.5, anti-CD27 conjugated to PE, anti-CD19 conjugated to PE-Alexa610 (all from Invitrogen/Caltag, Carlsbad, CA), anti-IgG conjugated to APC (BD Biosciences, San Jose CA), and anti-IgM conjugated to biotin (Southern Biotech, Birmingham, AL) followed by streptavidin-PE-Cy7 (Invitrogen/Caltag). The B cells were bulk sorted (CD3/CD20^{neg}, CD19^{low}, CD38^{high}, CD27$^{very high}$, IgG^{positive}) using a Becton-Dickinson FACS Aria cytometer (BD Biosciences, San Jose, CA) and then single cell sorted into 96-well PCR plates with a Cytomation MoFlo cytometer (Dako, Carpinteria, CA).

Single cell RT-PCR and PCR of antibody variable region genes

As detailed in (Smith et al., 2009), the plates receiving the single cells sorted as above contain 10 µl of a hypotonic buffer consisting of 10mM Tris-HCl with 40 U/µl RNase inhibitor (Promega, Madison, WI) in each well. After the sort, plates were immediately frozen on dry ice and stored at −80°C. A One-Step RT-PCR kit (Qiagen, Valencia, CA) was used to amplify V_H and V_K message using a cocktail of sense primers to the leader regions of each of the gene families and antisense primers to the constant regions of the heavy and kappa chains. Aliquots (1 µl) of the RT-PCR mixture were then amplified in separate heavy and kappa chain PCR reactions to obtain sequences, and separate aliquots (1 µl) were used for the final PCR reactions to incorporate restriction sites for further cloning. The amplified variable regions were then cloned into expression vectors (containing full length IgG$_1$ heavy or kappa constant regions), maxi-prepped (Roche, Indianapolis IN), and co-transfected into the HEK293A cell line using polyethyleneimine (PEI) (Polysciences, Warrington, PA). The transfected cells were allowed to secrete antibodies into serum-free DMEM supplemented with 1% Nutridoma (Roche, Indianapolis, IN) for five days. The secreted antibodies were then purified using protein A-agarose beads (Pierce, Rockford, IL). Antibody purity and integrity were verified by SDS-PAGE and concentrations were obtained with a Nanodrop spectrophotometer (Fisher, Pittsburg, PA).

Polysaccharide Affinity and avidity ELISAs

To screen for binding, ELISAs were first performed in plates coated with cocktails of five or six *S. pneumoniae* polysaccharides (ATCC, Manassas, VA), screening all 23 in this manner. Positive binders in this cocktail assay were then re-screened against each of the individual polysaccharides. As cell wall polysaccharide (CWPS) is an impurity in nearly all of the coat polysaccharides (Xu et al., 2005), antibodies that bound to all four groups were further tested on purified cell wall polysaccharide (Miravista Labs, Indianapolis, IN) at a 1:4000 dilution to confirm CWPS binding. Wells were coated with 10 µg of each polysaccharide (or total mixed polysaccharide), blocked with 20% FCS, and developed with anti-human IgG-HRP (Jackson ImmunoResearch, West Grove, PA) and Super Aqua Blue substrate (EBiosciences, San Diego CA). The absorbance was measured at 405nm on a microplate reader (Molecular Devices, Sunnyvale, CA). Antibody affinities (K_d) were calculated by curve fitting analysis of individual ELISA curves plotted from a dilution series of 16 two-fold dilutions of antibody beginning at 10 µg/ml. Binding curves were generated with a saturation binding, non-linear curve fit using GraphPad Prism software. Equilibrium dissociation constants (K_d) values for each hmAb were calculated using the equation $Y=B_{max}*X/(K_d+X)$ where B_{max} is the maximum number of binding sites, X is the concentration of the antibody and Y is the specific binding. Therefore the reported dissociation constants are equal to the concentration of antibody where half the binding sites

are occupied at equilibrium. Each antibody was run in duplicate in at least three unique experiments. The results for each experiment were then averaged to obtain the reported K_d.

For avidity ELISAs, one concentration of antibody was used (1 μg/ml) and an elution step was added before the addition of the conjugate. This elution step used various concentrations of ammonium thiocyanate (8 dilutions ranging from 3M to 0.06M) in PBS, as well as PBS alone. The percent of binding retained was calculated for each dilution of ammonium thiocyanate. These values were plotted versus thiocyanate concentration, and the concentration of thiocyanate resulting in 50% retention (or loss) of binding was calculated by fitting the data to a dose-response/sigmoidal curve with hillslope correction.

Opsonophagocytosis

Opsonophagocytosis assays (OPA) were performed by Flow Applications, Inc. (Okawville, IL) using the methodology of Martinez (Martinez et al., 2002). In brief, *S. pneumoniae* polysaccharides were covalently conjugated to fluorescent beads. HL60 cells, rabbit complement, and the polysaccharide beads, pre-incubated with antibody, were incubated together and fluorescence of the HL60 cells was measured at a variety of antibody concentrations by flow cytometry. OPA results are expressed as titers, with dilutions starting from 30μg/ml of antibody. Antibodies showing no facilitation of uptake were assigned a titer of 4.

Analysis of sequences and curve fitting

All curve fitting was performed using the GraphPad Prism software, with background subtraction or percent retention values calculated and averaged using Excel. Variable region sequences were analyzed using the International Immunogenetics Information System (IMGT, Montpellier, France, http://imgt.cines.fr/), as well as with in-house software and/or Vector NTI (Invitrogen, Carlsbad, CA). Clonally related antibodies were defined as those having the same VDJ/VJ usage in the heavy and light chains, respectively, and highly related $V_H D_H$, $D_H J_H$, and $V_K J_K$ junctions. CDR positions were used as defined by IMGT. Average nucleotide somatic hypermutation values were obtained by analyzing sequences (using IMGT) for the number of nucleotide changes from the germline in each antibody sequence. Resulting per-antibody values were then averaged to obtain average mutation rates per donor. The *n* value for these analyses included donors previously described (Wrammert et al., 2008): naïve cells from six donors (*n*=18, 42, 21, 34, 15, 36); IgM germinal center/memory cells from 17 donors (*n*=56, 158, 18, 91, 17, 10, 16, 30, 19, 28, 11, 36, 29, 13, 22, 20, 64); IgG germinal center/memory cells from 13 donors (*n*=110, 37, 19, 28, 174, 40, 25, 15, 21, 18, 22, 24, 19, 71); anti-influenza ASCs from 11 donors (*n*=63, 18, 33, 46, 49, 11, 36, 11, 30, 35, 25). The anti-polysaccharide ASC sequences are from the four donors in this study (PVAX1, 39; PVAX2, 49; PVAX3, 24; PVAX4, 25).

Results

Pneumovax23 induces a strong ASC response

Four individuals were immunized with Pneumovax23. Blood was drawn seven days post vaccination and PBMCs were isolated by Ficoll gradient. The cells were then stained and $CD38^{high}/CD27^{very\ high}$ ASCs were enumerated. Our previous results using these techniques after influenza vaccination (Wrammert et al., 2008) showed that this population represents an anamnestic ASC burst, which ranged from 1% to 16% of total peripheral blood B cells at day seven (average 6.4%). Pneumovax23 induces an even more robust ASC response (Fig. 1A): the ASC burst from one donor, PVAX2 represented 24.7% of peripheral blood B cells. As this was a primary vaccination for each donor, the average of 16.3% ASCs is impressive. This strong anamnestic response is likely due to the fact that *S. pneumoniae* is a ubiquitous

organism that causes both clinical and subclinical disease among the general population. ASCs were single cell sorted and cloned for making human monoclonal antibodies from antibody secreting cells. This technique has been previously described in detail (Smith et al., 2009; Wrammert et al., 2008; Smith et al., 2012). In total, including non-binding antibodies, 137 antibodies were produced and characterized (PVAX1, n=39; PVAX2, n=49; PVAX3, n=24; PVAX4, n=25).

Our previous study of the immune response to influenza vaccination (Wrammert et al., 2008) highlighted the strong clonality of the ASC response to that vaccine; the ASC response to immunization with Pneumovax23 was even more clonally restricted. Table I shows each clonal family, the number of clones isolated from each family, and the average affinity of the family, the serotype that the family binds, the OPA titer of one member, and VH and VK usage. We define the name of the clonal family by a representative antibody as described below.

The polysaccharide antibodies produced from ASCs are high affinity and are highly mutated resulting from an anamnestic response

Polysaccharide ELISA curves of individual antibodies against all serotypes and CWPS are shown in Fig. 2A. An arbitrary cutoff at an OD405 of 1.5 was used to separate high to moderate affinity antibodies from low to non-binding antibodies. Percentages were calculated using this cutoff as a means to determine the fraction of antibodies that had significant binding. Averaged across the four donors, 76% of the antibodies (PVAX1, 62%; PVAX2, 90%; PVAX3 75%; PVAX4, 75%) bound to *S. pneumoniae* serotype polysaccharide or cell wall polysaccharide from the vaccine (Fig. 2A).

As we described previously (Wrammert et al., 2008), the ASC recall response to the influenza vaccine is highly mutated, even more so than in a typical IgG germinal center memory cell. We hypothesized that this was due to the repeated nature of the annual vaccine, as well as frequent exposure to various influenza strains. We compared the frequency of mutations among different B cell populations using current and previously published data (see Fig. 2B). On average, the anti-polysaccharide ASCs examined in this study had accumulated a similar number of mutations as anti-influenza ASCs after seasonal influenza vaccination (Wrammert et al., 2008). This is particularly interesting because for each donor, this was a primary vaccination. If the donors were truly naïve to these polysaccharide antigens, the ASC response is predicted to have been smaller and the sequences of the antibodies would show less mutation. Thus, this vaccine likely elicited an anamnestic response which can only arise from previous infection or exposure to *S. pneumoniae* strains.

A large majority of polysaccharide antibodies produced from ASCs bind to a single serotype

Of the antibodies from the four donors that bound to polysaccharide (76% of the total), an average of 88% were serotype specific (Fig. 2C) (PVAX1, 88%; PVAX2, 90%; PVAX3, 94%; PVAX4, 80%). The observation that 88% of the antibodies produced by currently circulating ASCs specifically bound carbohydrate epitopes, discriminating between very closely related structures, reinforces the well-known specificity of the antibody repertoire.

Antibodies to structurally similar serotypes cross-react but may also be serotype specific

Although most of the antibodies studied were serotype specific, 12% of the antibodies we characterized bound two serotypes. Of the antibodies that bound two serotypes, several antibodies bound one pair of polysaccharides, 9N and 9V. These two carbohydrates have very similar non-branched structures, which differ only by a substitution of one of four D-

Glc in the 9N chain repeat with a D-Gal in 9V. Thus, it was not unexpected that some antibodies cross-reacted with both serotypes. However, we observed a wide variety of 9N and 9V binding antibodies, some of which cross-reacted and some that did not. For example, *PVAX1-p2D02* and *PVAX3-p1E01* antibodies were mono-specific for 9N and 9V, respectively (Fig. 3A), and showed significant OPA titers (256 and 512 respectively, Table I). *PVAX1-p4B03*, however, bound both serotypes, favoring 9N by five fold in both affinity and avidity (Fig. 3B), yet had identical OPA titers against each serotype (128). One antibody specific for 9N, *PVAX3-p1A03*, did not cross-react with 9V, but did cross-react with serotype 14 polysaccharide (Fig. 3C), with similar affinity and avidity, and exhibited an OPA titer against both serotypes, an observation that is difficult to explain only on the basis of carbohydrate sequence. Several of these cross-reacting antibodies were from the same donor, demonstrating a variety of antibodies to a specific serotype within a single individual. Serotypes 19A and 19F also have very similar structures differing in that 19F has a D-Glc in a 1–2 linkage and 19A has a 1–3 linkage. The antibody *PVAX4-p2D03* bound to both 19A and 19F with nearly equivalent affinities (Fig. 3D), although there was a four-fold difference in avidity (favoring 19A).

We also detected cross-reactivity between serotypes 17F and 33F (Fig. 4A and 4B) and 15B and 14 (Fig. 4C). While *PVAX4-p2G06* and *PVAX4-p2C04* were mono-specific for 17F and 33F, respectively (Fig. 4A), *PVAX4-p1C03* (from the same donor; Fig. 4B) cross-reacted with both serotypes. The affinity of *PVAX4-p1C03* for 33F was an order of magnitude better than the affinity for 17F, however, the avidities were similar. Of the antibodies shown in Figs 4A and 4B, only *PVAX4-p2G06* showed an OPA titer. The affinity of antibody *PVAX4-p1B01* was almost an order of magnitude higher for 15B than 14, although it showed a two-fold higher avidity for 14 and had identical OPA titers against each serotype. Overall, it was evident that although cross-reactions between two serotypes may be detected in serum, 85% of the actual antibodies making up this response are specific to only one polysaccharide. We encountered no antibodies that reacted with more than two serotypes with a measureable affinity or avidity.

Antibodies induced by CWPS impurities in the vaccine have similar sequences and do not facilitate opsonophagocytosis

Pneumovax23 is known to have CWPS, which is a carryover impurity from the purification of the polysaccharides (Xu et al., 2005). We were able to isolate antibodies to CWPS from three of the four donors. Including all members of clonal families, 13 of the 137 total antibodies characterized bound to CWPS (9.5%), including 9 of 44 from donor PVAX2 (20.4%). This is important to note because even though 7 of the 8 clonal families have better than nanomolar affinities (Fig. 5A), none of the CWPS clonal families showed OPA activity. Thus, 20.4% of the antibodies produced by PVAX2 are not capable of facilitating opsonophagocytosis and may be irrelevant bystanders produced by this impurity in the vaccine. Close examination of the sequences of the CWPS antibodies showed remarkable similarities even in unique donors (Fig. 5B). All seven of the high affinity families used VH3-30/33. PVAX1-p6D04 had low affinity (~40nM) and was the only member of the group not to use VH3-30 or VH3-33 (see Fig. 5B). These VH3 gene families are difficult to distinguish among highly mutated sequences, and even with significantly mutated HCDR1 and HCDR2, the antibodies encoded by the genes shown in Fig. 5B have similar affinities for CWPS. Kappa usage was also conserved with all CWPS antibodies using either VK4-1, VK1-5, or VK 1–16.

Each donor displays an anamnestic fingerprint of antibody serotype specificities of which 64% facilitate opsonophagocytosis

By displaying all four donors on a single histogram and reducing clonally related antibodies to a count of 1 (Fig. 6A), it is evident that the hmAbs isolated from each donor create a unique fingerprint with three donors binding 9V, 15B, and 17F, and only serotypes 8 and 33F being bound by all four donors. Also, no subject in the study produced an antibody that bound to serotypes 7F, 10A, or 12F.

The antibody clonal families detailed above are also shown in Fig. 6B and scored as either OPA+ (titer >4) or OPA- (titer =4). Overall, most clonal families show a 50% chance of facilitating opsonophagocytosis. Remarkably, all six clonal families that bind to serotype 8 and all four that bind to serotype 2 facilitate OPA, as well as all antibodies that bind to either 9N or 9V, whether or not they are specific to one serotype or cross-react with both. Serotypes for which no OPA-active antibodies were detected, with the exception of CWPS, are for the most part poorly represented in this study (serotypes 1, 11A, 14, 19A, and 23F).

Discussion

In this study, we analyzed the human IgG anamnestic response to Pneumovax23 immunization, on a per antibody basis, using antibody secreting cells (ASCs) that emerged seven days post vaccination as a source for the production of monoclonal antibodies. An analysis of these polysaccharide-specific monoclonal antibodies allowed a detailed study of the human antibody repertoire to this vaccine. This analysis also provided insight into the specificities and protective capacity of each antibody.

In an earlier study (Wrammert et al., 2008), we found that the magnitude of the anamnestic response following influenza vaccination was such that an average of 6% of total B cells were ASCs, yet some donors made poor to non-existent responses. Using these same techniques, some vaccines (notably Anthrax AVA) routinely result in very poor induction of a protective response (Smith et al., 2012). Here we report that Pneumovax23 invoked a two- to four-fold more robust response than the strongest responses induced in some of our influenza donors, suggesting that these polysaccharides are exceptionally efficient at triggering a memory response. Earlier studies (Nieminen et al., 1998; Nieminen et al., 1998; Clutterbuck et al., 2006) also detected antibody secreting cells seven days following vaccination with both the polysaccharide and conjugate vaccines, averaging over 100 serotype specific cells per million PBMCs. Our own ELISpot results were similar to these previous reports (data not shown), but the overall magnitude of the IgG ASC response as determined by flow cytometry was nonetheless surprising.

Our study has greatly increased the number of reported human monoclonal antibodies against *S. pneumoniae* that have been characterized with respect to binding and repertoire usage. These anti-polysaccharide antibodies are as highly mutated as antibodies that arise from repeated seasonal influenza vaccination. In comparing V gene usage in our antibodies to the previous reports, we observed similar trends. For example, Baxendale (Baxendale and Goldblatt, 2006; Baxendale et al., 2000) suggests that VH3-48 likely contributes to an antigen binding domain that prefers epitopes from serotypes 23F and 18C, as the two VH3-48 family antibodies they characterized bound those two serotypes. Similarly, Zhou found VH3-48 in a serotype 23F study (Zhou et al., 2002), but not a serotype 6B study (Zhou et al., 2004). Similarly, two of three VH3-48 clonal families (Table 1) characterized in our study also bind these two serotypes. We have also characterized a VH3-48 which bound serotype 2 (*PVAX2-p5E05*), a case of a VH3-48 binding a different serotype. We also observed remarkable similarity among antibodies which bound cell wall polysaccharide (CWPS) (Fig. 5B). Examining only the high affinity antibodies from seven clonal families

derived from two unique donors, we found that these antibodies use either VH3-30 or the closely related VH3-33. The CDR3s also showed remarkable similarity (*PVAX2-p6B03*, VKESATGWYRTADYW; *PVAX2-p5A06*, VKEYSWGYYRTADY*; PVAX4-p1A06*, VKEQGFGYYRTADY; *PVAX4-p1C04*, VKEQDYGYYRTADH). Thus, the chemical simplicity of repeated polysaccharide sequences seems to induce similar V gene family usage among unique individuals.

Although avidity has been shown to be an important correlate with protection (Anttila et al., 1999; Harris et al., 2007; Usinger and Lucas, 1999), thiocyanate ELISA is not commonly performed on monoclonal antibodies. We utilized it here because there are several complications in determining affinity by fitting simple ELISA curves. These include the magnified effects of small antibody concentration errors on affinities, uncertainty whether or not the antigen binding interaction is univalent or bivalent, and coating plates with large units of repeating epitopes. It is also possible that poly-reactive antibodies from SLE donors (and occasionally healthy controls) may interact with antigens outside of the binding site. These effects are minimized in the thiocyanate avidity ELISA system. Figs. 3D and 4C show data for antibodies which affinity and avidity ELISA binding measurements do not correlate. Both of these antibodies are from PVAX4 who is an SLE patient, and both antibodies are poly-reactive. We are currently exploring interesting antibodies such as these in more detail, but in these cases, thiocyanate avidity is a more reliable measure of the antibody-carbohydrate interaction.

Serum cross-reactivity is typically determined by depleting the serum with a particular serotype carbohydrate and then observing binding of the serotypes remaining in the serum. Soininen (Soininen et al., 2000), for example, found remarkable serum cross-reactivity to a variety of pneumococcal serotypes, especially in unvaccinated individuals. However, these assays require careful calibration, as well as pre-adsorption with CWPS and other polysaccharides to remove nonspecific reactivity, which is especially common in unvaccinated individuals (Marchese et al., 2006). Modern updates to this method using microarray printing and reading technology (Pickering et al., 2007), for example, have greatly improved the reliability of these assays; yet until our study, one could not be certain whether observed cross-reactivity was due to actual cross-reactive individual antibodies, or the polyclonal nature of serum antibodies.

Our study has addressed these ambiguities. Park (Park et al., 2009) describes cross-serotype monoclonal antibodies, deducing the common linear carbohydrate structure to which the antibodies were binding. Other reports (Baxendale and Goldblatt, 2006; Baxendale et al., 2000; Zhou et al., 2002; Zhou et al., 2004) do not specify cross-reactive antibodies, although those produced from Fab libraries were only panned with the serotype of interest. Our experiments characterized a large number of anti-pneumococcal human monoclonal antibodies, and although most of the antibodies are serotype specific, 12% were not. The explanation for the cross-reactivity of several of the monoclonal antibodies we characterized is clearly not as simple as finding similar primary polysaccharide structures. While 9N/9V and 19A/19F are structurally similar, 17F and 33F, and 14 and 15B do not have similar primary structures. Pickering (Pickering et al., 2007) found that 9V could inhibit 9N binding, 15B inhibited 14 binding, 19F strongly inhibited 19A binding and 33F strongly inhibited 17F binding, all matching our observed results (Fig. 3 and 4). Interestingly, the converse is not typically the case (14 does not inhibit 15B and 17F does not inhibit 33F), but this is likely an affinity issue. Using our results to illustrate this point, it is unlikely that *PVAX1-p4B03* binding to 9N could be inhibited by adding 9V polysaccharide because the affinity of *PVAX1-p4B03* for 9N is much higher than its affinity for 9V. Overall, we can say with confidence that a component of the serum cross-reactivity observed in these studies

is, indeed, due to individual monoclonal antibodies that bind to at least two different serotypes.

While the analysis of these human monoclonal antibodies helps to elucidate the basic anamnestic response, it may also serve a therapeutic purpose. As many of our current treatments can become ineffective due to antibiotic resistance, it is important to consider passive immunotherapeutics that can safely target pathogens. Several previous reports (Casal et al., 2002; Yuste et al., 2002) have explored the effects of specific antibodies in a mouse sepsis model. Remarkably, administering hyperimmune serum during an infection was able to reduce by eight-fold the amount of antibiotic required for the mouse to recover. This synergistic effect might be effectively used in treating difficult or invasive infections, such as empyema, or bacteremia in immunocompromised individuals. In addition to the myriad of treatment possibilities of fully human monoclonal antibodies, the drastically decreased risk of anaphylactic shock and of anti-treatment immune responses suggests that monoclonal antibody therapies may become as important in infectious diseases as they are currently in autoimmune settings.

In conclusion, the generation of hmAbs from ASCs seven days after vaccination with Pneumovax23 has given us greater insight into the anti-polysaccharide memory response in ways that conventional serology cannot. The overall anamnestic response is massive; the antibodies are predominantly serotype specific and facilitate opsonophagocytosis. Although this was a primary vaccination, the anamnestic character of the response indicates that the subjects were previously primed, presumably a result of the ubiquitous occurrence of *S. pneumoniae.* Furthermore, antibodies to CWPS have restricted V genes usage and do not facilitate opsonophagocytosis. In general, human monoclonal antibodies to the most widespread *S. pneumoniae* serotypes represent novel diagnostics for rapid serotyping and potentially passive immunotherapeutics for the treatment of resistant strains.

Acknowledgments

Abbreviations

ASC   antibody secreting cell

CWPS   cell wall polysaccharide

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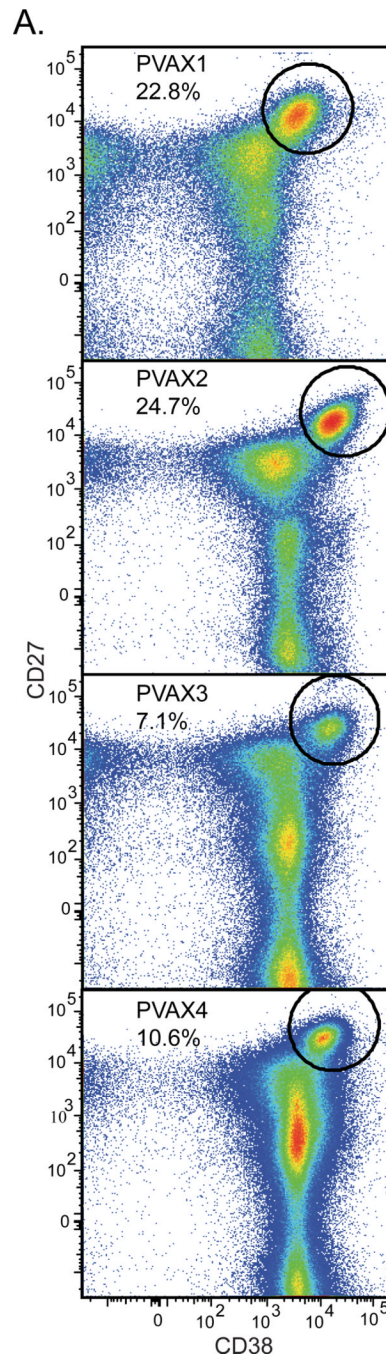
Figure 1. Pneumovax23 causes a massive ASC burst

A.) PBMCs were harvested from four donors seven days after vaccination with Pneumovax23. PBMCs were stained and sorted for cells which were CD3 and CD20 negative and CD19 intermediate. The dot plots presented indicate a large ASC burst in all four donors (CD27 very high, CD38 high; circular gate). Averaging the percentage of ASCs from these four donors, 16.3% of total B cells in the peripheral blood are ASCs.
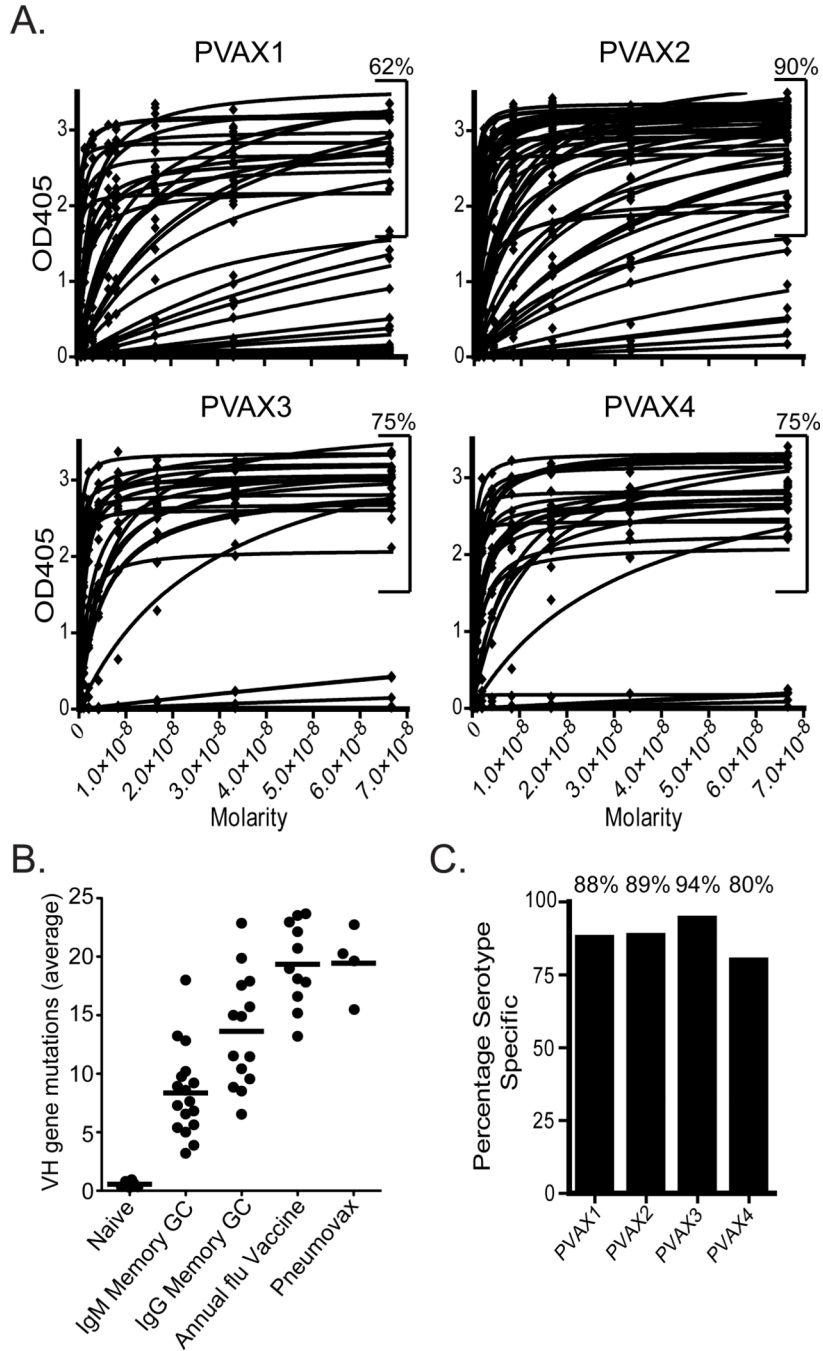
Figure 2. Binding, somatic hypermutation and serotype specificity of hmAbs from Pneumovax23
A.) An average of 76% (PVAX1, 62%; PVAX2, 90%; PVAX3, 75%; PVAX4, 75%) of the antibodies expressed bind to *S. pneumoniae* capsule or cell wall polysaccharide by ELISA. ELISA curves represent the binding of each antibody to a mixure of purified *S. pneumoniae* polysaccharides. B.) Each data point is the average frequency of somatic mutations (nucleotide) per sequence from each donor (*n* values in Methods). On average, the anti-polysaccharide ASCs had accumulated a similar number of mutations as anti-influenza ASCs after seasonal influenza vaccination (Wrammert et al., 2008). (GC are germinal center populations. The four averages of the Pneumovax23 donors are from the study, the others are from (Wrammert et al., 2008).) C.) The percentage of the total number of antibodies that

bind to a single serotype is plotted for each donor. While a significant percentage of antibodies are cross-reactive (12%), most of the antibodies produced are specific to a single serotype.
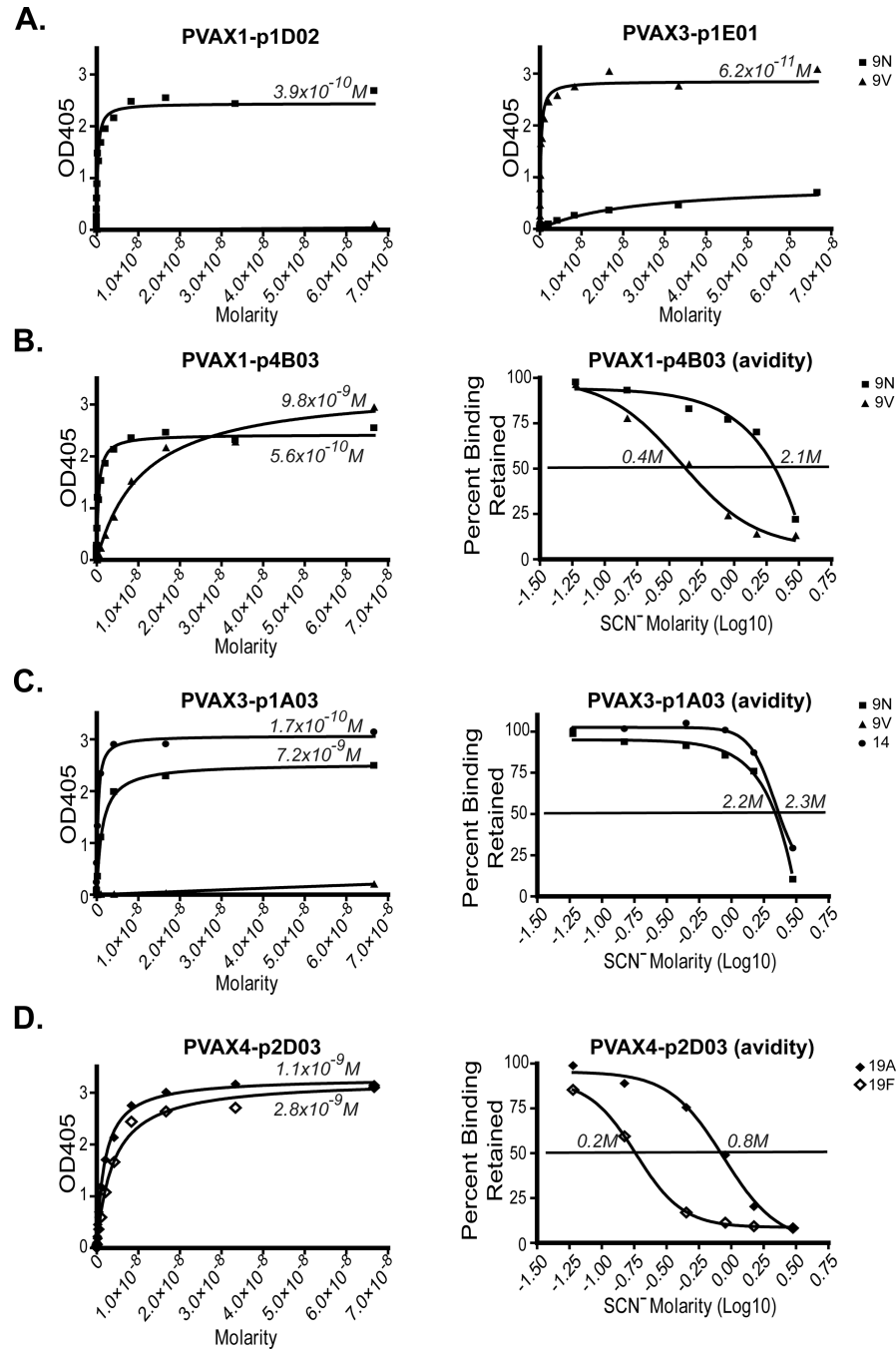
Figure 3. An individual can produce multiple antibodies to the same serotype, some of which are specific and others of which cross-react
A.) Binding of *PVAX1-p2D02* and *PVAX3-p1E01* to serotypes 9N and 9V. B.) Binding affinity and avidity measurements of *PVAX1-p4B03* to serotypes 9N and 9V. C.) Binding affinity and avidity measurements of *PVAX3-p1A03* to serotypes 9N, 9V, and 14. D.) Binding affinity and avidity measurements of *PVAX4-p2D03* to serotypes 19A and 19F. Affinities (K_d's) are expressed in molarity. The avidity is equal to the concentration of ammonium thiocyanate causing a 50% reduction (or retention) of binding.
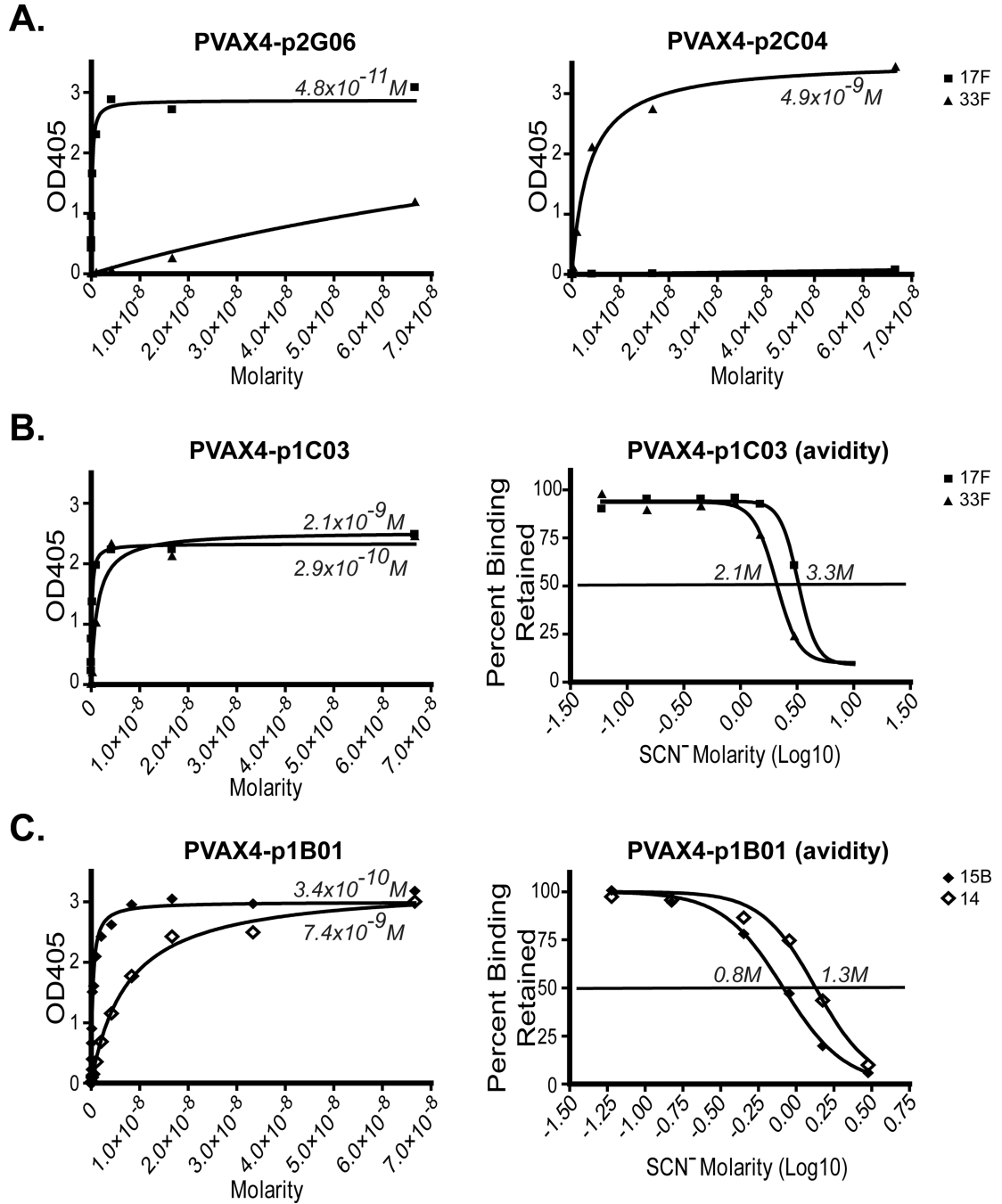
Figure 4. Characterization of cross-reactive antibodies to serotypes 15B and 14, and to 17F and 33F

A.) Binding of *PVAX4-p2G06* and *PVAX4-p2C04* to serotypes 17F or 33F. B.) Binding affinity and avidity measurements of *PVAX4-p1C03* to serotypes 17F and 33F. C.) Binding affinity and avidity measurements of *PVAX4-p1B01* to serotypes 15B and 14.

A.

B.

Name	V-HEAVY	J-HEAVY	HCDR1	HCDR2	HCDR3	V-KAPPA	J-KAPPA	KCDR1	KCDR2	KCDR3
PVAX1-p6D04	IGHV1-46	IGHJ4	GYTLTSYY	IRPT..DAST	AREVAAEGKAFDY	IGKV4-1	IGKJ4	QSLFYSSNKKNY	WAS	QQYYTPPLT
PVAX2-p3C04	IGHV3-30	IGHJ4	GFTFSNHG	ISYD..GSTK	AKGCSNGGNCFLIDY	IGKV4-1	IGKJ4	QSILSRSNNKNY	WAS	QQYYNAPLT
PVAX2-p5A06	IGHV3-30 or 33	IGHJ4	GFTLSTCG	TTYD..GDRK	VKEYSWGYYRTADY	IGKV1-5	IGKJ1	QSI......SSW	AVS	QQYSTYPWT
PVAX2-p6B03	IGHV3-30	IGHJ4	GFTLSSCG	STYDGDGNQK	VKESATGWYRTADY	IGKV1-5	IGKJ1	ETL......NNW	EAS	HQYNKYPWT
PVAX2-p7D03	IGHV3-30	IGHJ6	GFIFSSNG	ISSD..GSRR	AKGCSGENCFYMDD	IGKV4-1	IGKJ4	QTVLSSSNNKNY	WAS	QQCYNAPLT
PVAX4-p1A06	IGHV3-30 or 33	IGHJ4	GFTLSSCG	ITYD..GRSH	VKEQGFGYYRTADY	IGKV1-5	IGKJ1	QSI......NSW	EAS	HQYDKYPWT
PVAX4-p1C04	IGHV3-30	IGHJ5	GFTLSTCG	KTYD..GREE	VKEQDYGYYRTADH	IGKV1-5	IGKJ1	QSI......NAW	KAS	QQYDKYPWT
PVAX4-p2F01	IGHV3-33	IGHJ4	GFTFSRYG	IWHD..GSNT	LRGNPPSSPTDY	IGKV1-16	IGKJ4	QGI......NNY	STS	QQYNSYPLT

Figure 5. Binding and V gene usage analysis of CWPS antibodies
A.) Binding affinity measurements of representative antibodies from each clonal family to
CWPS. B.) Variable heavy and kappa gene usage for each of the CWPS clonal families. Red
amino acids in the H and KCDRs indicate a mutation from germline.
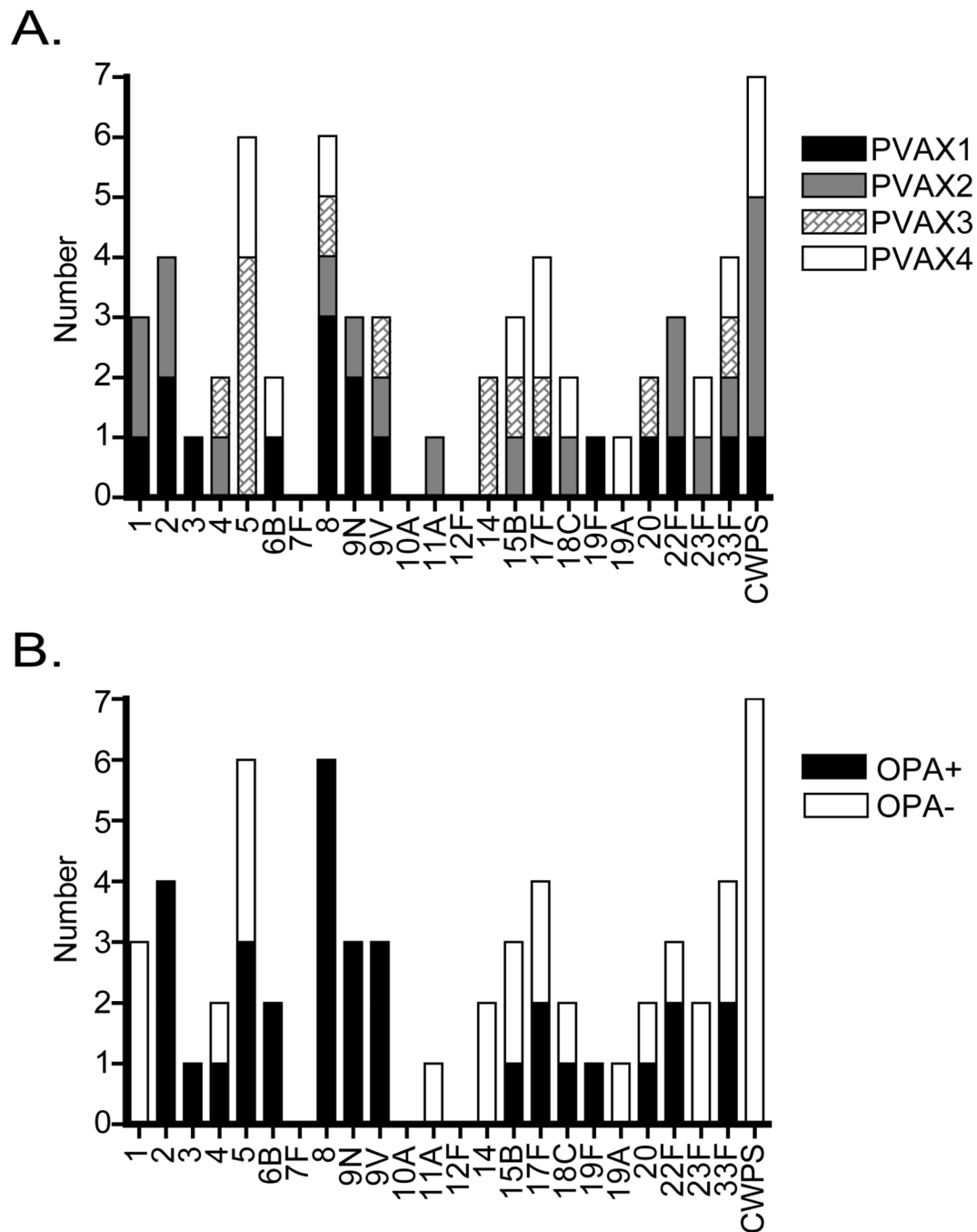
Figure 6. Analysis of the "anamnestic fingerprint" of each donor and opsonophagocytic titer of each clonal family

A.) Histogram showing serotype specificity fingerprint for all clonal families from all four donors. B.) Histogram showing the opsonophagocytic potential of each clonal family. Open bars are OPA negative (titer = 4), black bars are OPA positive (titer > 4).

Table 1

Summary of *S. pneumoniae* polysaccharide antibodies.

Antibody	Number of Clones	Serotype(s)	Kd (M)^1	OPA^2	VH gene	JH gene	Heavy CDR3	VK gene	JK gene	Kappa CDR3
PVAX1-p2 C01	3	20	1.1E-08	512	VH3-66	JH6	AKGVTSFDY	VK3-20	JK4	QQFGSSPPDT
PVAX1-p2 C04	1	1	2.2E-10	none	VH3-23	JH4	ARDPGIRNGMGV	VK2-30	JK1	MQVTHWPRT
PVAX1-p2 D02	1	9N	3.9E-10	256	VH3-23	JH4	AKAHRGDWNNFFDY	VK3-11	JH4	QQSGDWPLT
PVAX1-p2 D03	1	19F/19A	1.2E-08	1024 / none	VH4-59	JH3	AREWSGFDF	VK3-20	JK1	QQYGSLPRT
PVAX1-p2 E01	3	8	7.7E-11	512	VH3-7	JH4	ARGQWLAF	VK2-30	JK2	MQGTHWPYT
PVAX1-p3 C02	1	2	1.4E-10	4096	VH3-7	JH4	ARGRNNFRH	VK1-33	JK3	QQFESFPRT
PVAX1-p3 C03	1	22F	1.8E-10	32	VH3-66	JH4	ARELGVFHSGGDQWLGPLDC	VK3-15	JK3	HQYKNWPPMGT
PVAX1-p3 G01	2	2	1.4E-10	2048	VH3-49	JH4	RWTGGVSFGAY	VK1-5	JK1	QQYDIYLT
PVAX1-p3 G06	1	8	2.1E-08	16	VH3-74	JH4	ARDYYHSVDY	VK2-30	JK2	MQGTHWPYT
PVAX1-p4 B01	2	33F	4.0E-08	256	VH4-59	JH4	ARGPDAHKTGY	VK4-1	JK1	QQYAATPWT
PVAX1-p4 B03	1	9N/9V	5.6E-10	128 / 128	VH3-74	JH4	ARDSYTSPDY	VK2-30	JK4	MQGSHWPLT
PVAX1-p4 C01	1	8	9.5E-10	128	VH3-15	JH3	TTDNGVKAFDI	VK4-1	JK3	HQYYTTPFA
PVAX1-p4 G01	1	6B	3.1E-10	256	VH3-74	JH4	TRGGSGATINY	VK1-39	JK4	QQSHSSPLT
PVAX1-p6 C01	1	9V	3.0E-08	256	VH4-61	JH4	ARDRAGIDGYNYYFDY	VK1-5	JK2	QQYYSFYT
PVAX1-p6 D04	1	CWPS	4.2E-08	none	VH1-46	JH4	AREVAAEGKAFDY	VK4-1	JK4	QQYTPPLT
PVAX1-p6 E03	1	3	8.9E-10	128	VH3-7	JH3	ARGQSYPGI	VK3-15	JK1	QQYNNWPRT
PVAX1-p6 E06	1	17F/33F	9.4E-09	8 / none	VH4-59	JH4	AGRAYSSGYYYLIDY	VK3-15	JK2	QHYHNWPPT
PVAX2-p3 C04	3	CWPS	7.9E-11	none	VH3-30	JH4	AKGCSNGGNCFLIDY	VK4-1	JK4	QQYYNAPLT
PVAX2-p3 C05	1	4	1.8E-10	256	VH3-23	JH3	AKGGYYESGTMRAFDI	VK3-11	JK4	QQRSNWPAT
PVAX2-p3 F03	2	2	1.5E-10	4096	VH3-7	JH4	ARGESNFRY	VK1-33	JK3	QQFVSFPRT
PVAX2-p3 G05	9	18C	2.8E-10	64	VH3-7	JH4	ARDSTSPARFGY	VK3-20	JK2	QHYGTSPPRYT
PVAX2-p4 B03	1	1	3.4E-08	none	VH3-53	JH4	ATGGMTSSWYGY	VK4-1	JK2	QQYYSTPYT
PVAX2-p4 C02	5	9N/9V	2.7E-10	512 / 8	VH1-46	JH4	SMGPPYCTGGSCYSACDF	VK3-20	JK2	QRYGNSPPYT
PVAX2-p4 D06	5	9V	2.6E-10	2048	VH3-15	JH5	TTDIGKGWTHYPDL	VK4-1	JK4	LQYRSAPFT
PVAX2-p5 A06	2	CWPS	5.1E-10	none	VH3-30	JH4	VKEYSWGYYRTADY	VK1-5	JK1	QQYSTYPWT
PVAX2-p5 B06	3	1	1.4E-10	none	VH3-74	JH4	ARSPGGYFDY	VK3-15	JK1	QQYSTWLWT
PVAX2-p5 C04	1	8	2.3E-08	32	VH3-15	JH4	TTDDLKN	VK1-39	JK2	QQRYRIPYS
PVAX2-p5 E05	1	2	2.8E-10	2048	VH3-48	JH6	ARGRDCYGGNCVIYFHYYGLDV	VK2-28	JK2	MRALQTPYT

Antibody	Number of Clones	Serotype(s)	Kd (M)[1]	OPA[2]	VH gene	JH gene	Heavy CDR3	VK gene	JK gene	Kappa CDR3
PVAX2-p6 B03	3	CWPS	6.4E-11	none	VH3-30	JH4	VKESATGWYRTADY	VK1-5	JK1	HQYNKYPWT
PVAX2-p6 C05	1	33F	3.3E-09	none	VH3-66	JH3	ARDIPTTFGIGEAFDI	VK1-5	JK1	QQYYSWGT
PVAX2-p6 G04	1	22F	4.4E-10	128	VH1-46	JH4	ARDDSAFDY	VK2-24	JK1	MQASQSTWT
PVAX2-p7 D03	1	CWPS	1.8E-09	none	VH3-30	JH6	AKGCSGENCFYMDD	VK4-1	JK4	QQCYNAPLT
PVAX2-p8 B01	1	22F	2.3E-08	none	VH1-46	JH4	TREIGAVVVDATSLGWLGYFDY	VK3-15	JK1	QQYNNWPPVT
PVAX2-p8 B05	2	15B	1.7E-10	none	VH3-7	JH4	AGWGRTQD	VK2-30	JK2	MQYTFWPHT
PVAX2-p8 E03	1	23F	3.3E-08	none	VH3-30	JH3	TKEGAPPGKYAFDI	VK3-11	JK3	QHRGEWPPGAT
PVAX2-p8 F05	1	11A	1.8E-10	none	VK3-72	JH3	LKDSSQYSFDA	VK1-9	JK4	QQFKGYPLT
PVAX3-p1 A02	3	5	5.1E-10	1024	VH4-59	JH4	ARGDGYNFF	VK1-9	JK2	QQINSYPRT
PVAX3-p1 A03	1	14/9N	1.7E-10	512 / 32	VH3-30	JH5	AKCGAEDSTTVWLNWFDP	VK3-11	JK4	QQRADWPLT
PVAX3-p1 B05	3	5	9.5E-10	none	VH3-23	JH4	AKPNYFGSGSPDY	VK3-11	JK2	LQCSNWPMYT
PVAX3-p1 C04	1	5	2.8E-10	2048	VH4-59	JH4	VKEQDYGYYRTADH	VK1-6	JK2	QQYDKYPWT
PVAX3-p1 E01	2	9V/9N	6.2E-11	512/256	VH3-20	JH3	VRVAVPAATYTRGNDAFDI	VK1-17	JK1	LQHSSFPWT
PVAX3-p1 F02	1	14	1.0E-09	none	VH3-15	JH4	TTAHGPVGDH	VK4-1	JK5	QQYYTTPSIT
PVAX3-p1 G05	1	15B	1.6E-10	none	VH3-7	JH4	ARAGGCSSTRCHTTPGFDY	VK4-1	JK5	QQYYTTPPIT
PVAX3-p2 A02	1	5	1.4E-10	512	VH4-39	JH3	ASLSGTNAFDI	VK3-11	JK1	QQRSSGRT
PVAX3-p2 D04	1	8	7.4E-09	256	VH3-23	JH4	AKPRGYSYGYFDY	VK3D-20	JK2	QQYGISPRT
PVAX3-p3 A02	1	17F	2.7E-09	none	VH3-7	JH4	APPARRLDY	VK2-29	JK1	MQGTHHPWT
PVAX3-p3 A04	1	4	3.8E-08	none	VH4-74	JH4	ARSNAGHEA	VK4-1	JK4	QQYYSTPLT
PVAX3-p3 B03	1	20	1.5E-09	none	VH1-46	JH4	ARDIPHANLDY	VK1-17	JK1	LQHTTFPWT
PVAX3-p3 C03	1	33F	1.1E-09	128	VH3-23	JH4	VKDRVPPGDVPGDF	VK3-11	JK5	QQRRTWPPLT
PVAX4-p1 A01	2	23F	2.5E-09	none	VH3-48	JH6	ATLLLRDNQLDV	VK2-30	JK1	MQGTHWRT
PVAX4-p1 A06	1	CWPS	7.9E-10	none	VH3-33	JH4	VKEQGFGYYRTADY	VK1-5	JK1	HQYDKYPWT
PVAX4-p1 B01	2	15B/14	2.0E-10	256 / 256	VH4-59	JH3	ARRNDFNI	VK3-20	JK3	QQYGSSPFT
PVAX4-p1 C03	1	17F/33F	2.9E-10	none	VH3-23	JH4	SIWWGTSVQYPLVLDY	VK3D-15	JK5	QQYSKWPPIT
PVAX4-p1 C04	1	CWPS	2.0E-09	none	VH3-30	JH5	VKEQDYGYYRTADH	VK1-5	JK1	QQYDKYPWT
PVAX4-p1 D02	5	5	2.0E-10	none	VH4-61	JH4	ARGHGFNAY	VK3-20	JK1	QQYGNSPRT
PVAX4-p1 D04	1	6B	8.8E-11	512	VH3-15	JH4	TTVRNMADLSLNH	VK3-20	JK1	QQYDDSRWT
PVAX4-p2 A01	1	18C	4.2E-09	none	VH3-48	JH4	ATGNRGSLPRR	VK2D-28	JK2	MQALRSPYT
PVAX4-p2 C04	1	33F	4.9E-09	none	VH3-7	JH4	VRDGWDTFFDS	VK2-30	JK2	MQGRYWPYT

Antibody	Number of Clones	Serotype(s)	Kd (M)[1]	OPA[2]	VH gene	JH gene	Heavy CDR3	VK gene	JK gene	Kappa CDR3
PVAX4-p2 D03	1	19A/19F	1.1E-09	none / 8192	VH3-74	JH4	VNFQLG	VK3-20	JK1	QQYGNSPRT
PVAX4-p2 E04	1	8	5.1E-10	1024	VH3-30-3	JH5	ARAEYCSPGDCFLIDT	VK2-30	JK1	MQGTHWRT
PVAX4-p2 F01	1	CWPS	9.6E-10	none	VH3-33	JH4	LRGNPPSSPTDY	VK1-16	JK4	QQYNSYPLT
PVAX4-p2 G01	1	5	1.4E-09	none	VH3-23	JH6	AKVVYSRPPMDV	VK1D-39	JK1	QQSYSTPWT
PVAX4-p2 G06	1	17F	4.8E-11	128	VH3-7	JH4	ARASRETGEPY	VK2-30	JK1	MQATHWPWT

[1] reported ELISA affinities are the averaged affinities of all of the clones in the clonal family

[2] reported OPA titers are for one representative member of each clonal family (a titer = 4 is reported as "none")